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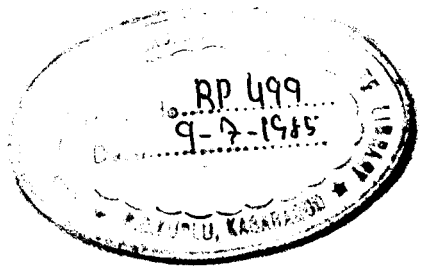

Rhizobium strain effects on yield and bleeding sap amino compounds in *Pisum sativum*

Lis Rosendahl

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Bleeding sap composition, dry matter production and nitrogen distribution in pea (*Pisum sativum* L. cv. 'Bodil') grown with and without nitrate and nodulated with either *Rhizobium leguminosarum* strain 128c53 or strain 1044 were compared. Nitrate increased the total dry matter production of both symbioses, but decreased both the proportions of below-ground dry matter to total dry matter production and nodule dry matter to total below-ground dry matter production. The total dry matter yield and N-accumulation was greater in the symbiosis with strain 1044, whereas the accumulation of N in the roots plus nodules relative to the total N-accumulation was greater with strain 128c53 due to a higher production of nodule tissue. The root bleeding sap of the symbiosis with the greater yield (strain 1044) contained high levels of asparagine and aspartic acid. In the 128c53 symbiosis, glutamine plus homoserine accounted for a higher percentage of the organic solutes transporting newly assimilated nitrogen from the root system than in the association with 1044. The *Rhizobium* strain effect on amino compound composition of the bleeding sap may indicate an influence of the bacteroids on either the N-assimilatory enzyme system in the plant cytosol, or on the pools of the Krebs cycle intermediates or related compounds in the nodules.

Additional key words – Aspartyl compounds, glutamyl compounds, nitrate.

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Introduction

Energy is considered to be the main limiting factor of the symbiotic N_2-fixation in legume nodules (Hardy and Havelka 1975), which may utilize up to about one-third of the total net photosynthate produced by the host plant (Schubert and Ryle 1980). Besides energy for growth and maintenance the nodules use ATP and reductant for the reduction of N_2 to ammonia and for assimilation of the latter, which also utilizes carbon skeletons (Atkins et al. 1978).

The overall costs of the N_2-fixation vary considerably for different symbioses (Atkins et al. 1978, Layzell et al. 1979, Schubert and Ryle 1980). The *Rhizobium* strains may influence the cost of the N_2-fixation through different carbohydrate requirements for the establishment and functioning of the symbiosis (Gibson 1966). Such strain effects may be ascribed to varying nodule respiration (Rainbird et al. 1983, Witty et al. 1983). Besides, the efficiency of nitrogenase and the presence of hydrogenase activity in the bacteroids may influence the N-accumulation of the host plant (Lepo et al. 1981, Schubert and Evans 1976). Furthermore, strains of *Rhizobium* may influence the bleeding sap composition of peas, and the presence of asparagine coincided with high N-accumulation in the tops (Wieringa and Bakhuis 1957). In pea plants solely dependent on nitrogen from cotyledons and symbiotic N_2-fixation, asparagine did not appear in the bleeding sap before leghaemoglobin became visible in the root nodules. Thereafter, asparagine remained the dominant N-compound of the bleeding sap (Pate and Wallace 1964).

The present work provides additional quantitative information on the effect of *Rhizobium* strains on bleeding sap composition of N-compounds in pea – *R. leguminosarum* symbioses.

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Materials and methods

Growth conditions

Pea (*Pisum sativum* L. cv. 'Bodil') plants were maintained in a growth chamber under a 16/8 h light/dark cycle at 17/15°C, 70–75% relative humidity. Alternating rows of mercury lamps (HPLR, 250 W, Philips) and incandescent lamps (75 W, Philips) provided a photon flux density of approximately 370 μmol m^{-2} s^{-1} measured in the photosynthetically active range (400–700 nm) with an LI-185B quantum sensor (Lambda Instruments). Seeds within the weight range 0.30 to 0.32 g were surface-sterilized with 70% (v/v) ethanol for 2 min, and rinsed thoroughly with sterile water. After drying, the seeds were treated with the fungicide 'Thiram' (2 g 'Thiram'/kg seed). Six seeds were sown in an irradiation-sterilized (10 MeV electron beam, 4 Mrad) growth medium consisting of a 2:1 (v/v) sand: gravel mixture in a modified 'Leonard jar' pot system (Skøt 1983). Immediately before sowing, the surface of the growth medium was inoculated with a three-day old YMB (Yeast Mannitol Broth) suspension culture of *Rhizobium leguminosarum*. One set of pots was inoculated with *R. leguminosarum* strain 128c53 (originally obtained from Dr. J. Burton, Nitragin Company, Milwaukee, WI., USA); another set of pots was inoculated with *R. leguminosarum* strain 1044 (originally obtained from Rothamsted Experimental Station, Harpenden, Herts., U.K.). Nitrate was added to the nutrient solution in half of the pots from each *Rhizobium* treatment, which resulted in four treatments: 2 *Rhizobium* strains ± NO_3^-. The pots were arranged in a randomized block design with five replicate pots of the four treatments for each harvest. After emergence, seedlings were thinned to four per pot, and the surface of the growth medium was covered with a layer of gravel. Nutrient solutions were added to the reservoirs as needed and contained 0.7 m*M* $Ca(H_2PO_4)_2$, 0.8 m*M* $MgSO_4$, 0.41 m*M* K_2HPO_4, 0.06 m*M* $FeSO_4$, 0.12 m*M* Na_2 EDTA, 48.5 μ*M* H_3BO_3, 10.1 μ*M* $MnSO_4$, 0.7 μ*M* $ZnSO_4$, 0.32 μ*M* $CuSO_4$, 0.31 μ*M* Na_2MoO_4, and 0.21 μ*M* $CoCl_2$. One solution also contained 10 m*M* KNO_3, while an equivalent amount of potassium was added as K_2SO_4 to the N-free solution. Both solutions were adjusted to pH 6 with K_2HPO_4. Actual acidity in the pots during growth was about pH 7 due to a high $CaCO_3$ content in the sand. Samples of nutrient solutions were taken from the reservoirs before each watering to ensure that the nitrate concentration was maintained.

Sampling and analyses

Seedlings emerged 4 days after sowing. Data were collected 31 and 42 days after seedling emergence, that is during vegetative growth and early flowering, respectively. Three of the plants from each pot were used for bleeding sap collection. At both harvests the collection of bleeding sap was initiated 4.5 h after the start of the photoperiod to reduce diurnal variability (Pate 1962). Plants were detopped with a scalpel at the lower epicotyl, 1–2 cm above the gravel, and the cut end was rinsed with distilled water and blotted dry. Sap was collected in volume-marked micropipettes (50, 100 or 200 μl, Blaubrand) fastened to the cut ends with Tygon tubing (R 3603). Sampling time was 30 min at the first harvest but had to be extended to 90 min at the second due to a lag-period before production of bleeding sap. The bleeding sap volumes were recorded, and bleeding sap pooled from each pot was immediately stored at −20°C. The last plant from each pot was used to obtain some information on the levels of C_2H_2-reduction and H_2-evolution (Bethlenfalvay and Phillips 1978). Furthermore, the number, weight, and N-content of the nodules were recorded. The total dry matter production per pot was recorded after drying for 24 h at 80°C. The nitrogen content of plant material was measured with a Technicon Autoanalyser after sulphuric acid:salicylic acid NO_3^--reduction and Kjeldahl digestion. Samples of bleeding sap were adjusted to pH 2.2, and the amino acid content analysed by separation on a Beckman Amino Acid Analyser 120 C modified with an automatic sampling loader and a Data Transfer Unit with a teleprinter for calculating peak areas. AA-15 resin and lithium citrate buffers for physiological fluid analysis were used for the neutral and acid amino compounds. In this amino acid separation technique the peaks of glutamine and homoserine coincide. However, their specific absorbances are equal, which allowed a recording of their sum. NO_3^- in the bleeding sap and in nutrient reservoirs of the pots was determined by the diphenylamine method (Feller et al. 1971). The concentration of ureides (allantoin and allantoic acid) in the bleeding sap was measured using a differential analysis (Vogels and Van Der Drift 1970).

Results

Dry matter production

NO_3^- increased the dry matter production of both above- and below-ground biomass (Tab. 1). In contrast, NO_3^- decreased the proportions of below-ground dry matter to total dry matter production and nodule dry matter to total below-ground dry matter production. The total dry matter production was greater in the 1044 symbiosis than in association with 128c53. This strain effect was expressed mainly in a higher dry matter production of tops, whereas below-ground dry matter production was significantly increased only at 42 days. At both harvests, however, the proportion of below-ground biomass to total dry matter production was higher in the 128c53 symbiosis than in the association with 1044 (Tab. 1). This was due mainly to a larger production of nodule biomass in the former symbiosis regardless of NO_3^- treatment.

Tab. 1. Dry matter production of *Pisum* in symbiosis with either *Rhizobium* strain 128c53 or 1044 with or without nitrate ($\pm NO_3^-$). ***, $P \leq 0.001$; **, $0.001 \leq P \leq 0.01$; *, $0.01 \leq P \leq 0.05$; ns, not significant.

Treatment		Days after seedling emergence									
		31					42				
		Total (g/pot)	Top (g/pot)	Root + nodules g/pot	(% of total)	Nodules (% of root + nodules)	Total (g/pot)	Top (g/pot)	Root + nodules g/pot	(% of total)	Nodules (% of root + nodules)
$-NO_3^-$	128c53	3.8	2.5	1.3	(34)	34	7.3	5.5	1.8	(25)	33
	1044	4.6	3.3	1.3	(27)	12	12.4	10.0	2.4	(20)	9
$+NO_3^-$	128c53	6.7	5.1	1.6	(24)	15	19.2	16.5	2.7	(14)	9
	1044	8.2	6.6	1.7	(20)	4	22.8	19.7	3.1	(14)	3
Analysis of variance											
NO_3^-		***	***	***	***	***	***	***	***	***	***
Rhizobium strain		*	*	ns	***	***	***	**	*	**	***
NO_3^- × *Rhiz.* strain		ns	ns	ns	ns	*	ns	ns	ns	*	***

Tab. 2. Nitrogen content of *Pisum* in symbiosis with either *Rhizobium* strain 128c53 or 1044 with or without nitrate ($\pm NO_3^-$). See Tab. 1; †, $0.05 \leq P \leq 0.10$.

Treatment		Days after seedling emergence									
		31					42				
		Total (mg/pot)	Top (mg/pot)	Root + nodules mg/pot	(% of total)	Nodules (% of root + nodules)	Total (mg/pot)	Top (mg/pot)	Root + nodules mg/pot	(% of total)	Nodules (% of root + nodules)
$-NO_3^-$	128c53	149	98	51	(35)	59	270	197	73	(27)	61
	1044	189	149	39	(21)	29	462	388	75	(17)	28
$+NO_3^-$	128c53	290	225	64	(23)	32	707	602	105	(15)	21
	1044	359	303	56	(16)	10	790	674	116	(15)	9
Analysis of variance											
NO_3^-		***	***	***	***	***	***	***	***	***	***
Rhizobium strain		*	**	*	***	***	**	**	ns	***	***
NO_3^- × *Rhiz.* strain		ns	ns	ns	*	†	ns	†	ns	***	**

The mean dry weight per nodule was more than 3 times as high in the 128c53 symbiosis as in the association with 1044. There was a tendency to a higher nodule number in the 1044 symbiosis, but the overall nodule biomass production was about 3 times as high in the 128c53 symbiosis as in the association with 1044.

Nitrogen accumulation

Nitrogen concentrations in the different tissues were similar in the various treatments, although the 1044 symbiosis tended to contain a higher nitrogen concentration in tops at the first harvest and in nodules at 42 days. Thus, the overall *Rhizobium* effect on nitrogen content in the different tissues was in the same order of magnitude as the effect of *Rhizobium* on dry matter production (Tab. 2). The highly significant effect of the strain of *Rhizobium* on the nitrogen distribution within the host-nodule association (Tab. 2) was due mainly to differences in production of nodule tissue, which in both symbioses contained a 3-fold higher N-concentration than the root tissue less nodules.

Bleeding sap composition

The N-transporting solutes of the bleeding sap listed in Tab. 3 accounted for 86% or more of the recorded N-export compounds on a molar basis (Fig. 1), and the organic compounds listed are considered the main transporters of fixed N_2. At 31 days the main organic N-solutes of the bleeding sap occurred in the highest concentrations in the 1044 symbiosis (Tab. 3). However, this *Rhizobium* effect was only significant concerning aspartic acid and asparagine. The effect of

Tab. 3. Main nitrogen transporting solutes in the bleeding sap (m*M*), and bleeding sap exudation rate (μl plant^{-1} h^{-1}) of *Pisum* in symbiosis with either *Rhizobium* strain 128c53 or 1044 with or without nitrate ($\pm NO_3^-$). See Tab. 1; †, $0.05 \leq P \leq 0.10$. a) Hse, homoserine.

Treatment		Days after seedling emergence									
		31					42				
		Asp	Asn	Gln + Hse[a]	NO_3^-	(Exudation rate)	Asp	Asn	Gln + Hse[a]	NO_3^-	(Exudation rate)
$-NO_3^-$	128c53	5.8	14.4	13.5	0.0	(15)	13.2	24.9	32.4	0.0	(19)
	1044	9.1	24.9	14.9	0.0	(24)	15.6	36.1	18.9	0.0	(13)
$+NO_3^-$	128c53	2.4	7.3	6.2	15.8	(136)	3.7	10.0	6.4	18.1	(56)
	1044	3.5	10.7	6.5	15.3	(133)	3.7	8.2	6.3	19.5	(99)
Analysis of variance											
NO_3^-		***	***	***	***	***	***	***	***	***	***
Rhizobium strain		***	**	ns	ns	ns	ns	†	*	ns	†
NO_3^- × *Rhiz.* strain		*	†	ns	ns	ns	ns	*	*	ns	*

Rhizobium strain on glutamine plus homoserine concentration was negligible and, therefore, glutamine plus homoserine accounted for a higher percentage of the N-transporting solutes in the symbiosis with 128c53 than in the one with 1044 (Fig. 1). This was particularly obvious at 42 days in the $-NO_3^-$-treated plants. At this time the concentration of glutamine plus homoserine in the bleeding sap of the 128c53 symbiosis had exceeded that of the 1044 symbiosis under $-NO_3^-$-conditions, whereas aspartic acid and asparagine still occurred at the highest concentrations in association with 1044 (Tab. 3). Thus, in the 1044 symbiosis asparagine plus aspartic acid amounted to 69%, while glutamine plus homoserine accounted for only 25% of the N-transporting solutes (molar basis). In the association with 128c53, the corresponding values were 51 and 43% (Fig. 1).

The NO_3^--concentration in the bleeding sap of the NO_3^--fed plants was high and accounted on a molar basis for 37 to 48% of the N-transporting solutes. The highly significant effect of NO_3^- on the concentrations of the main N-transporting solutes in the bleeding sap (Tab. 3) must be considered in the light of the large differences in the amounts of bleeding sap obtained. Thus, the amounts of bleeding sap obtained in the $+NO_3^-$-treatments were approximately 7 and 5 times larger than the corresponding records from the $-NO_3^-$-treatments at 31 and 42 days, respectively (Tab. 3).

At 31 days NO_3^- did not alter the relative composition of organic N-solutes in the bleeding sap and, therefore, the influence of the *Rhizobium* strain on transport solutes seemed to be independent of the nitrogen status of the host plant (Fig. 1). In contrast, at 42 days, this strain effect appeared to be influenced by the nitrate treatment. However, in NO_3^--fed symbioses N-compounds are passed to the xylem from two distinct sources via different plant tissues, and, therefore, their relative contribution to the bleeding sap may depend on the exudation rate. This was significantly greater in the

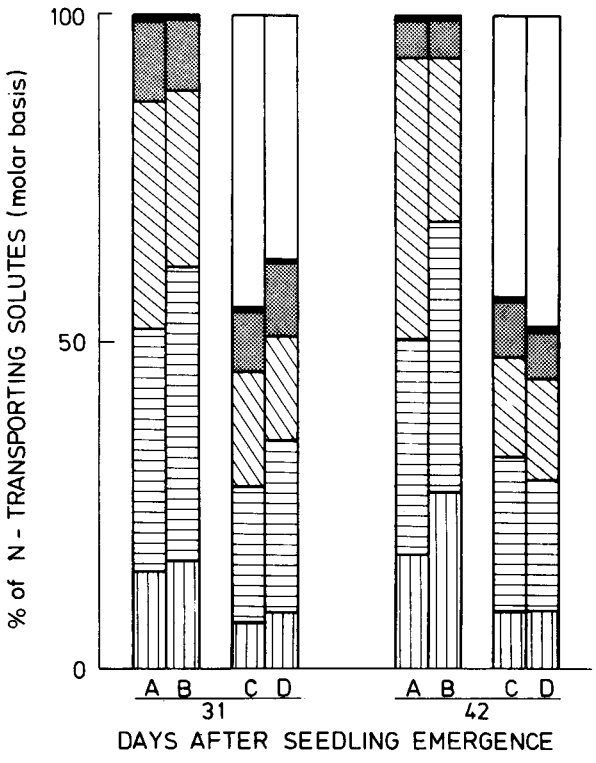

Fig. 1. Relative content of N-transporting solutes in the bleeding sap of *Pisum* in symbiosis with either *Rhizobium* strain 128c53 or 1044 with or without nitrate ($\pm NO_3^-$). A, 128c53 $-NO_3^-$; B, 1044 $-NO_3^-$; C, 128c53 $+NO_3^-$; D, 1044 $+NO_3^-$. ▥, aspartic acid; ▤, asparagine; ▧, glutamine + homoserine; ▩, other amino acids (see text) and ammonia; ■, ureides; □, NO_3^-.

1044 than in the 128c53 symbiosis at 42 days in the $+NO_3^-$-treatments (Tab. 3), and hence, a direct comparison of the strain effect on the relative composition of N-transporting solutes might not be valid in the NO_3^--fed symbioses at this time.

Concerning other organic N-solutes of the bleeding sap not included in Tab. 3 ureides were found at significantly higher levels in the $-NO_3^-$-treatments, but concentrations never exceeded 0.7 m*M* and no effect of *Rhizobium* strain was recorded. Serine and alanine were found at higher concentrations in the 1044 than in the 128c53 symbiosis, but never exceeded 1 m*M*. Glutamic acid, threonine, methionine, isoleucine, leucine, lysine, histidine, and arginine never exceeded 0.6 m*M* and concentrations were not influenced by *Rhizobium* strain. Glycine, valine, and cystine occurred only in trace amounts. Tyrosine and phenylalanine were never detected. Ammonia was recorded in low concentrations (<0.9 m*M*), which probably originated from minor post harvest breakdown of solutes.

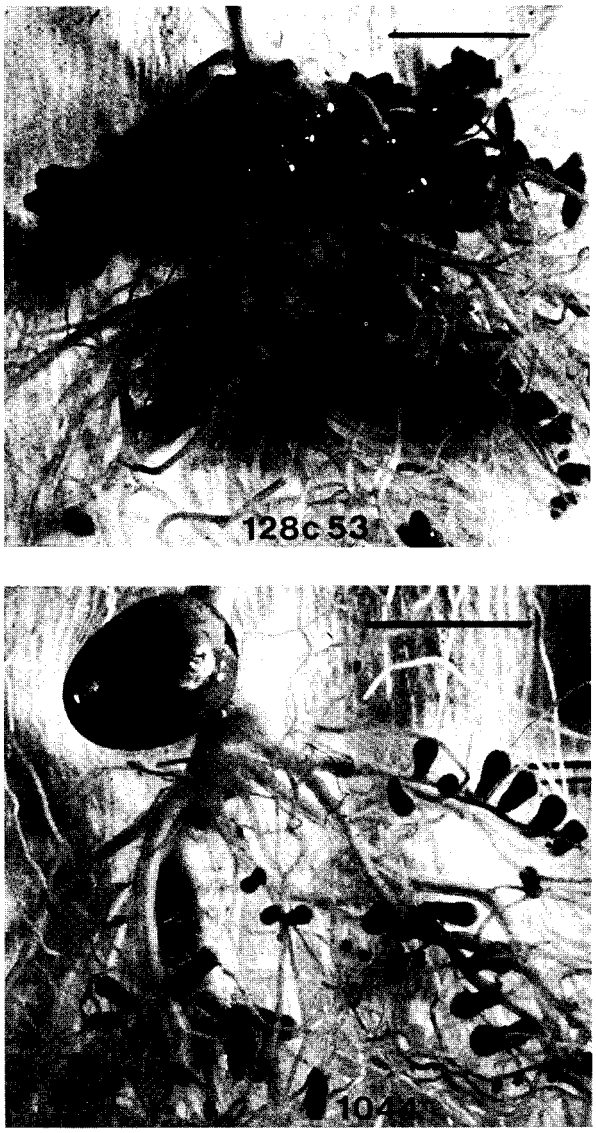

Fig. 2. Root nodules of *Pisum sativum* cv. 'Bodil' in symbiosis with *R. leguminosarum*. Note the different nodule morphology: meristematic regions are multiple in 128c53 nodules (a), but single in 1044 nodules (b). Bars represent 1 cm.

Discussion

The recorded surplus of aspartyl compounds in the bleeding sap of the most effective symbiosis (in terms of total N-accumulation) is consistent with the qualitative results by Wieringa and Bakhuis (1957). The N_2 fixed in the bacteroids is exported as ammonia and assimilated in the plant cytosol via the glutamine synthetase glutamate synthase cycle (Boland et al. 1980). The induction of glutamate synthase activity in the plant cytosol of the nodules by nitrogenase activity has been indicated in soybean symbioses (Sen and Schulman 1980). The possibility of regulation through induction and expression of nodule-specific host genes affecting the assimilatory enzyme system cannot be excluded (Fuller et al. 1983). Furthermore, ATP and Krebs cycle intermediates are required for the action of the assimilatory enzymes (Boland et al. 1980). In the present study, the small amount of asparagine plus aspartic acid and the high amount of glutamine plus homoserine in the bleeding sap of the 128c53 symbiosis compared with that in the 1044 symbiosis may, therefore, reflect an influence of the bacteroids on either the assimilatory enzyme system in the plant cytosol or on the pools of the Krebs cycle intermediates or related compounds in the nodules.

A surplus of exportable aspartyl- over glutamyl-compounds is likely to involve the activity of a phosphoenol pyruvic acid carboxylase system in the roots and nodules as a mechanism of generating oxaloatate in addition to the Krebs cycle (Pate 1977). Therefore, aspartyl compounds in the bleeding sap are considered to indicate a better carbon economy of the nodules than glutamyl compounds (Pate 1977). The subsequent membrane transfer and release of export products to the xylem from the nodule symplast does involve ATPase activity, but the costs should be relatively small and differences in the form of solutes are unlikely to have major effects on the overall C budget of the nodule (Layzell et al. 1979).

In nodulated pea plants fed with 10 m*M* NO_3^- the relative dependence on NO_3^--N is about 50% (C. G. O. Oghoghorie 1971. Thesis, The Queens Univ., Belfast, U.K.). The nitrate reductase of the roots is saturated at about half the NO_3^- concentration in the rooting medium (Oghoghorie and Pate 1971) and this may explain the high NO_3^--concentrations found in the bleeding sap (Tab. 3).

The *Rhizobium* effects on root-shoot ratios and on N-distribution within the plants were observed also in

the NO_3^--fed plants (Tabs 1 and 2) and were, therefore, unlikely to have originated strictly from differences in N-availability of the plants. The differences in nodule dry weight contributed considerably to the strain effect on N-distribution within the plants. Both the number, size, and external form of the nodules differed for the investigated symbioses. The nodules of the 128c53 symbiosis tended to form more meristematic areas on each nodule, whereas the nodules of the 1044 symbiosis were regular and club-shaped (Fig. 2). Thus, the morphogenesis of the nodules seemed to differ for these symbioses, which may result in varying hormonal and energetic interactions between the microsymbiont and host plant.

Both the C_2H_2-reduction rate and hydrogenase activity are higher in the 128c53 symbiosis with this host variety than in the association with 1044 (R. Wyndaele, personal communication). The data obtained in the present work on C_2H_2-reduction and H_2-evolution were in agreement with those observations (the data are not presented here). A possibility of conserving energy through hydrogen metabolism exists when the membrane-bound hydrogenase in the bacteroids is coupled to an ATP-forming respiratory chain (Nelson and Salminen 1982). However, in the present symbioses the lowest overall N-accumulation was recorded in the symbiosis with the highest C_2H_2-reduction rate and hydrogenase activity (strain 128c53).

The recorded differences in biomass distribution and bleeding sap composition may reflect *Rhizobium* strain-determined deficiences in the 128c53 symbiosis, and this strain effect seems to be of importance to the overall N-accumulation. A reflection of strain-determined deficiencies on the bleeding sap composition may occur in other pea varieties nodulated with other *Rhizobium* strains. The present experiment confirms that a significant *Rhizobium* strain effect on bleeding sap composition exists and this may deserve further attention.

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